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Geographically Distinct Expression Profile of Host Defense Peptides in the Skin of the Chinese Odorous Frog, *Odorrana margaretae*

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Abstract *Odorrana margaretae* (Anura: Ranidae) is widely distributed in the southern provinces of China. Previously, 72 antimicrobial peptides (AMPs) belonging to 21 families were identified from the skin of *O. margaretae*, which were captured in the Hunan province. In the present study, five *O. margaretae* frogs were captured from the Guizhou province and a total of 28 cDNAs encoding 17 host defense peptides (HDPs) belonging to 14 families were cloned from the skin cDNA library of *O. margaretae*. Among the 17 HDPs, only one (brevinin-1-Omar5) had been characterized. The distinct HDP expression profiles for *O. margaretae* in the previous and present study may be attributed to the environmental differences between the sampling locations and the genetic divergence among *O. margaretae* populations. Besides, 11 of the 17 HDPs identified in the present study were novel for ranids. In order to understand their roles in host defense reactions, three HDPs (odorranain-H-OM1, odorranain-M-OM and ranatuerin-2-OM), which possess low sequence similarity with the known amphibian HDPs, were selected for further chemical synthesis and functional analysis. Odorranain-H-OM1 showed direct antimicrobial activity against bacteria and fungi. Odorranain-M-OM exhibited concentration-dependent anti-oxidant activity. Ranatuerin-2-OM showed lectin-like activity and could strongly hemagglutinate human intact erythrocytes with or without the presence of Ca^{2+}. The diverse activities of HDPs implied that they may play different roles in host defense reactions of *O. margaretae*.

Keywords amphibian, host defense peptides (HDPs), *Odorrana margaretae*, antimicrobial activity, anti-oxidant activity, erythrocytes hemagglutinating activity

1. Introduction

Host defense peptides (HDPs) are gene-encoded innate immune effectors found in nearly all sorts of organisms (Choi *et al.*, 2012; Hancock and Sahl, 2006). Being rich in arginine and lysine residues, HDPs are generally positively charged, so are also termed as being cationic host defense peptides (Mookherjee and Hancock, 2007; Yeung *et al.*, 2011). HDPs usually possess direct antimicrobial activity against a variety of invading pathogens, such as bacteria, fungi, viruses and protozoa. Besides, some of them are critical effectors of both innate and adaptive immunity, participating in diverse immune modulating processes, such as inflammation, wound repair, chemoattractant and tumorigenesis (Auvynet and Rosenstein, 2009; Hancock and Sahl, 2006). As a result, they are regarded as potential anti-infective therapeutic agents of direct-acting antimicrobial drugs or immunomodulating agents (Hancock and Sahl, 2006).

Over the past several decades, extensive studies have revealed that amphibian skin secretions are rich resources of bioactive compounds, especially HDPs (Bevins and Zasloff, 1990). So far, a large number of HDPs have been identified from amphibians. Amphibian derived HDPs possess diverse activities, such as antimicrobial, protease inhibitory, cytotoxic, antioxidant, histamine-releasing and lectin-like activities (He *et al.*, 2012; Li *et al.*, 2008b; Li *et al.*, 2008a; Yang *et al.*, 2009). To date, more than one thousand HDPs have been identified from the skin

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of ranid frogs (Conlon *et al.*, 2004; Li *et al.*, 2007; Yang *et al.*, 2012). They differ in size, charge, structure and activity and accordingly are divided into many different families. However, the structures of their precursors are very similar, including an N-terminal signal peptide domain, an acidic spacer peptide domain and a C-terminal mature peptide domain (Mechkarska *et al.*, 2010). The signal peptide domain and acidic spacer peptide domain are highly conserved, which implies that they may derive from an identical ancestral gene (Conlon, 2011).

Odorrana is a genus belonging to the "true frogs" (Ranidae) living in Southeast Asia and surrounding regions. There are 18 kinds of odorous frogs all over the world and 13 of them live in China. Previous studies indicated that odorous frog skin secretions possess the most abundant HDPs among amphibians. Using biochemical and molecular biological methods, 107 novel AMPs belonging to 30 different families were identified from the skin of *O. grahami* (Li *et al.*, 2007). 728 AMPs belonging to 97 different families were identified from 9 Chinese odorous frogs, of which 662 AMPs were novel (Yang *et al.*, 2012). *Odorrana margaretae*, a species endemic to China, is widely distributed in the southern provinces, including Hubei, Hunan, Sichuan, Guizhou and Guangxi. Previously, Yang *et al.*, (2012) collected *O. margaretae* frogs from Hunan province and identified 72 AMPs belonging to 21 families from the skin, in which 62 were novel (Yang *et al.*, 2012). In the present study, we report the collection of *O. margaretae* frogs from Guizhou province and molecular cloning of 17 HDPs belonging to 14 families from the skin. The results indicate distinct HDP expression profile in the skin of *O. margaretae* collected from Hunan and Guizhou province and possible reasons are discussed. Subsequently, three HDPs were selected for chemical synthesis and their *in vitro* activities were determined.

2. Materials and Methods

2.1 Collection of frogs Adult specimens of *O. margaretae* (n = 5, weight range 39.00 ± 6.67 g) were captured in October from Libo (107°37′ E, 25°21′ N), a county in the southern part of the Guizhou province. The climate in Libo was humid subtropical monsoon and the temperature when sampling was 15–25 ˚C. After collecting, the frogs were quickly killed with a needle and the dorsal skins were removed and stored in liquid nitrogen until use. All the animal experimental protocols were approved by the Animal Care and Use Ethics Committee of Guizhou Normal University.

2.2 cDNA library construction and screening of cDNAs encoding HDPs The stored dorsal skin was ground into powder in liquid nitrogen and total RNA was extracted using Trizol reagent (Life Technologies, CA, USA). cDNA library was constructed using a In-Fusion SMARTer^{TM} Directional cDNA Library Construction Kit (Clotech, Palo Alto, CA, USA). First-strand cDNA synthesis was carried out using SMARTScribe^{TM} Reverse Transcriptase (Clotech) and SMARTer V Oligonucleotide and 3′ IF SMARTer CDS Primer. Second-strand cDNA synthesis was performed by a long-distance PCR method using Advantage 2 Polymerase Mix (Clontech) in the presence of 5′ PCR Primer II A and 3′ IF SMARTer PCR Primer. The synthesized second-strand cDNAs was used as template for the following PCR-based cDNAs screening.

According to the conserved signal peptide domain of previously characterized HDPs from ranid frogs, a sense oligonucleotide primer (5′-CCCCATGTTCACCTTGAAG-3′) was designed and coupled with 3′ antisense primer (5′-TACGCGACGCGATACGCGAAT-3′) designed according to the sequence of 3′ IF SMARTer CDS Primer to screen the HDP encoded cDNAs. The PCR procedure was: 5 min of denaturation at 94 ˚C; 30 cycles: denaturation at 94 ˚C for 30 s, primer annealing at 56 ˚C for 30 s, extension at 72 ˚C for 1 min. The PCR product was purified by gel electrophoresis, cloned into pMD19-T vector (Takara, Japan) for sequencing.

2.3 Bioinformatic analysis The deduced mature peptides of HDPs were analyzed through the protein blast item of Basic Local Alignment Search Tool in NCBI (http://www.ncbi.nlm.nih.gov/). Secondary structures of the selected HDPs were predicted using The PSIPRED Protein Structure Prediction Server provided by Bioinformatics Group of UCL Department of Computer Science (http://bioinf.cs.ucl.ac.uk/psipred/).

2.4 Peptide synthesis The selected HDPs (odorranain-H-OM1, odorranain-M-OM and ranatuerin-2-OM) for further functional analysis were synthesized by solid phase synthesis on an Applied Biosystems model 433A peptide synthesizer according to the manufacturer's standard protocols. After cleavage and deprotection of side-chain, the crude peptides were purified on a Vydac C18 RP-HPLC column (25 cm × 1 cm). Elution was carried out at a flow rate of 1 ml/min by a linear gradient of acetonitrile in 0.1 % trifluoroacetic acid in water. Identity of the synthetic peptides was confirmed by automated Edman degradation method and MALDI-TOF-

MS analysis. Peptides containing two cystein residues were further subject to oxidation to form an intrapeptide disulphide bridge. The purity of the synthetic peptides was confirmed to be higher than 95 %. The synthetic peptides were dissolved in sterile deionized water to an ultimate concentration of 2 mg/ml and stored at −20 °C.

2.5 Antimicrobial assay Ten strains of standard and clinic-isolated microorganisms, including Gram-positive bacteria, Gram-negative bacteria and fungi, were used in antimicrobial assay. Minimal inhibitory concentrations (MICs) of the peptides were determined by a standard 2-fold microdilution method in 96-well microtiter plate as described previously (Lu *et al.*, 2010). Briefly, microorganisms were incubated in Mueller-Hinton broth (MH) at 37 °C to exponential phase and diluted with fresh MH broth to 10^6 CFU/ml. 50 μl of serial dilutions of peptides in MH broth were prepared in 96-well microtiter plates and mixed with 50 μl of diluted bacteria inoculum. Plates were incubated at 37 °C for 18 h and the minimal concentration at which no visible growth occurred was recorded. Ampicillin was used as a positive control and the assay was conducted in triplicate.

2.6 Anti-oxidant assay Anti-oxidant activity of the peptides was determined using two free radical scavenging methods, 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic Acid) ($ABTS^{·+}$) radical scavenging assay.

DPPH radical scavenging assay was conducted according to the previously described method (Yang *et al.*, 2009). DPPH (Sigma, USA) was dissolved in methanol to a final concentration of 6×10^{-5} mol/L. 92 μl DPPH solutions were mixed with 8 μl peptide solutions of serial concentrations. After 30 min incubation at room temperature, the amount of reduced DPPH was quantified by measuring a decrease in absorbance at 520 nm.Deionized water was used as negative control. Inhibition of free radical by DPPH in percentage (I %) was calculated according to the formula:

$$I \%= (A_{blank}-A_{sample}) \times 100/A_{blank}$$

$ABTS^{·+}$ radical scavenging assay was carried out according to the previously described method with slight modification (Liu *et al.*, 2010). ABTS (Amresco, USA) was dissolved in phosphate buffered saline (PBS, 8.18 g NaCl, 0.27 g KH_2PO_4, 3.58 g $NaHPO_4 \cdot 11H_2O$ and 0.15 g KCl in 1 L H_2O, pH 7.4) to prepare 2 mmol/L ABTS stock solution. $ABTS^{·+}$ was produced by mixing ABTS stock solution and 70 mmol/L $K_2S_2O_8$ solution with a volume ratio of 250 : 1. The mixture was left in dark at room temperature for 15–16 h and diluted with PBS to absorbance of 0.80 ± 0.03 at 734 nm before use. 48 μl $ABTS^{·+}$ solutions were mixed with 2 μl peptide solutions of serial concentrations. The mixtures were incubated at room temperature for 10 min and the absorbance at 734 nm was measured. Deionized water was used as negative control. Percentage of $ABTS^{·+}$ inhibition (I %) was calculated according to the formula:

$$I \%= (A_{blank}-A_{sample}) \times 100/ A_{blank}$$

2.7 Erythrocyte hemagglutination assay Fresh human erythrocytes were collected from a donor and stored in Alsever’s solution (8.0 g sodium citrate, 0.55 g citric acid, 20.5 g glucose and 4.2 g NaCl in 1 L H_2O, pH 6.1) to prevent coagulation. Assay was performed in U-well microtiter plates (96 wells) according to the method described by Li et al (Li *et al.*, 2008b). Human fresh erythrocytes were washed twice with TBS buffer (6.06 g Tris base and 5.84 g NaCl in 1 L H_2O, pH 7.5) or TBS+Ca^{2+} buffer (6.06 g Tris base, 5.84 g NaCl and 1.12 g $CaCl_2$ in 1 L H_2O, pH 7.5) and resuspended with the same buffer to a final concentration of 2 % (v/v). 10 μl peptide solutions (2 mg/ml) was mixed with 90 μl erythrocyte solutions in U-well microtiter plate. The plate was incubated quietly at room temperature for 45 min and the result was observed. Deionized water was used as negative control and the assay was conducted in triplicate.

3. Results

3.1 cDNA cloning and deduced mature peptides characterization A total of 28 cDNAs encoding precursors of 17 different HDPs were cloned from *O. margaretae* skin cDNA lilbrary (GenBank Accession: KC427241-427268). Sequences of the 17 HDP precursors were shown in Figure 1. All of them share similar structural organizations, including an N-terminal 22 residue signal peptide sequence, an acidic residue-rich spacer peptide domain, a conserved dibasic protease cutting site (K-R or N-R) and a C-terminal mature peptide domain. The deduced 17 mature HDPs belong to 14 different families (Figure 1). 6 of the 17 HDPs, including odorranain-A6, odorranain-B6, odorranain-O-RA, brevinin-1-Omar5, esculentin-1-RA2 and esculentin-2-RA2, have already been identified in other frogs. The other 11 HDPs were novel and a primary structure comparison of the 11 novel HDPs with related peptides from other ranid frogs were carried out. As shown in Figure 2, the 11 HDPs showed high similarities with

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                       ←—— 1 ——→              ←—— 2 ——→              3 ←—— 4 ——→
Preproishikawain-7-OM  MFTLKKTLLLLFFLGTISLTLC EEERDADEEENEVSGYAS-NVDI KR CGYRHGRAHCGRG
Preproranatuerin-2-OM  MFTLKKSLLLLFFIGTISLSLC KQERSADEDEGEVIE-----QEV KR GLMNTASNVLTNVAGTVMDKIKCRFTGGC
Prepropalustrin-OM     MFTLKKSLLLLFFIGTISLSLC EQERDADEDEGEALE------EV KR GLWNTIKQAGKKFFLSVLDKIRCKVAGGCRT
Preproandersonin-Q-OM  MFTLKKSLLLLFFLATINLSLC QEERNAEEER-RDDQDKMDVEME KR FLPAAICSVIKTC
Preproodorranain-A6    MFTLKKSLLLLFFLGTISLSLC EQERDAEEEEGSENGAE--DIKI NR VVKCSYRPGSPDSRCK
Preproodorranain-A-OM  MFTLKKSLLLLFFLGTISLSLC EEERDADEEGSEENGAEAKVEDI KR RVFKCYKPDSRGFQICE
Preproodorranain-B6    MLTLKKPLLLLFFLGIISLSFC EQERDADEDDG----GEVTEEEV KR AALRGCWTKSIPPKPCSGKR
Preproodorranain-C-OM1 MLTLKKSLLLLFFLGTISLSLC QEERDADEDEG-----EMTEEEV KR SVMGTVKDLLIGAGKSAAQSVLKALSCKLSKDC
Preproodorranain-C-OM2 MFTLKKSLLLLFFLGTISLSLC QEERDADEDEG-----EMTEEEV KR SVMGTVKDLLIGAGKSAAQSVLKSLSCKLSNDC
Preproodorranain-H-OM1 MFTLKKSLLLLFFLGTINLSLC QDETNAEEE--RRDEEVAKMEEI KR GILGNILGMGKKVLCGLSGLC
Preproodorranain-H-OM2 MFTMKKSMLLLFFLGTISLSLC EQERNADEEE-RRDEEVAKMEEI KR GILGNIVGMGKKIVCGLSGLC
Preproodorranain-M-OM  MFTLKKFLLLLFFLGIVSSSPC LRKRDADEEGNEENGGEAKLEDI KR ATALVLPPRGLLPIGFKFKDIILCRKD
Preproodorranain-O-RA  MFTLKKSLLLLFFLGTINLSLC EEERGAD----EEDGGEAKLEDI KR AVPLIYNRPGIYVTKRPKGK
Preproodorranain-P-OM  MFTLKKSLLLLFFLGTINLSLC QEERNADEE---ERRDERDAEVE KR VIPFVASVAAEMMQHVYCAASKRC
Preproodorranain-Q-OM  MFTLKKSLLLLFFLGTISLSLC EEERDADEE-----RRDDEVEET KR APFRMWYMYHKLKDMEPKPVA
Preproesculentin-1-RA2 MFTLKKPLLLIVLLGIISLSLC EQERAADED---------EGSEI KR GLFSKFAGKGIKNLIFKGVKNIGKEVGMDVIRTGIDVAGCKIKGEC
Preproesculentin-2-RA2 MLTLKKSLLLLFFLGTISLSFC QEERAADEE------DNGEVEEV KR GIFTLIKGAAKLIGKTVAKEAGKTGLELMACKITNQC
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Figure 1 Multiple alignments of the 17 preproHDPs cloned from the skin cDNA library of *O. margaratae*. (1) the signal peptide domain; (2) the acidic residue-rich spacer peptide domain; (3) the dibasic proteases cutting site; (4) the putative mature peptide domain.

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Ishikawain-7-OM    CGYRHGRAHCGRG                     13  O. margaretae  92%
Ishikawain-7       CGYRHGRLNCGRG                     13  O. ishikawae
                          **
Ranatuerin-2-OM    GLMNTASNVLTNVAGTVMDKIKCRFTGGC     29  O. margaretae  86%
Ranatuerin-2P-RA   GFMNTVSNVLTNVAGTVKDKIKCKFTGGC     29  O. andersonii
                    *    *             *      *
Palustrin-OM       GLWNTIKQAGKKFFLSVLDKIRCKVAGGCRT   31  O. margaretae  94%
Palustrin-OG2      GLWDTIKQAGKKFFLNVLDKIRCKVAGGCRT   31  O. grahami
                      *           *
Andersonin-Q-OM    FLPAAICSVIKTC                     13  O. margaretae  92%
Andersonin-Q       FLPAAICLVIKTC                     13  O. andersonii
                          *
Odorranain-A-OM    RVFKCYKPDSRGFQICE                 17  O. margaretae  94%
Odorranain-A8      RVFKCYKPDSRGFQVCE                 17  O. grahami
                                 *
Odorranain-C-OM1   SVMGTVKDLLIGAGKSAAQSVLKALSCKLSKDC 33  O. margaretae  91%
Odorranain-C-OM2   SVMGTVKDLLIGAGKSAAQSVLKSLSCKLSNDC 33  O. margaretae  94%
Odorranain-C7      GVLGTVKDLLIGAGKSAAQSVLKTLSCKLSNDC 33  O. grahami
                   * *                     *
Odorranain-H-OM1   GILGNILGMGKKVLCGLSGLC             21  O. margaretae  81%
Odorranain-H-OM2   GILGNIVGMGKKIVCGLSGLC             21  O. margaretae  86%
Nigrosin-2Sc       GILSNVLGMGKKIVCGLSGLC             21  O. schmackeri
                      * **      **
Odorranain-M-OM    ATALVLPPRGLLPIGFKFKDIILCRKD       27  O. margaretae  74%
Odorranain-M3      ATALGLSSRGLLPIGFMFKDTIRCRKY       27  O. grahami
                       * **        *   * *
Odorranain-P-OM    VIPFVASVAAEMMQHVYCAASKRC          24  O. margaretae  96%
Brevinin-1Ra       VIPFVASVAAEMMQHVYCAASRRC          24  P. ridibundus
                                        *
Odorranain-Q-OM    APFRMWYMYHKLKDMEPKPVA             21  O. margaretae  95%
Odorranain-Q1      APFRMWYMYHKLKDMEPKPMA             21  O. grahami
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Figure 2 Comparison of the primary structures of the 11 novel HDPs deduced from *O. margaratae* with related HDPs from other ranid frogs. Sequences used in this figure were derived from NCBI protein database. Length and origin of the HDPs were shown on the right. Percentages represented the maximal sequence similarity. Asterisks (*) indicated variable residues.

related peptides, with a sequence identity of 74 %–95 %. Among which, odorranain-H-OM1, odorranain-M-OM and ranatuerin-2-OM exhibited relatively low sequence similarity, therefore, they were selected for further chemical synthesis and functional analysis. Physical and chemical parameters of the three selected HDPs, including theoretical pI, molecular weight, net charge and grand average of hydropathicity, were computed by Compute pI/Mw (http://web.expasy.org/compute_pi) and ProtParam (http://web.expasy.org/protparam/) and listed in Table 1. Besides, secondary structures of the three HDPs were also predicted. As shown in Figure 3,

Table 1 Physical and chemical parameters of the three selected HDPs from *O. margaretae.*

Peptide	GRAVY	Number of amino acids	Net charge	Theoretical pI	Mw
Odorranain-H-OM1	1.171	21	2+	8.9	2044.58
Odorranain-M-OM	0.533	27	3+	9.78	2995.71
Ranatuerin-2-OM	0.29	29	2+	8.9	3000.53

GRAVY: grand average of hydropathicity. Structural parameters of each peptide calculated using the ProtParam tool.

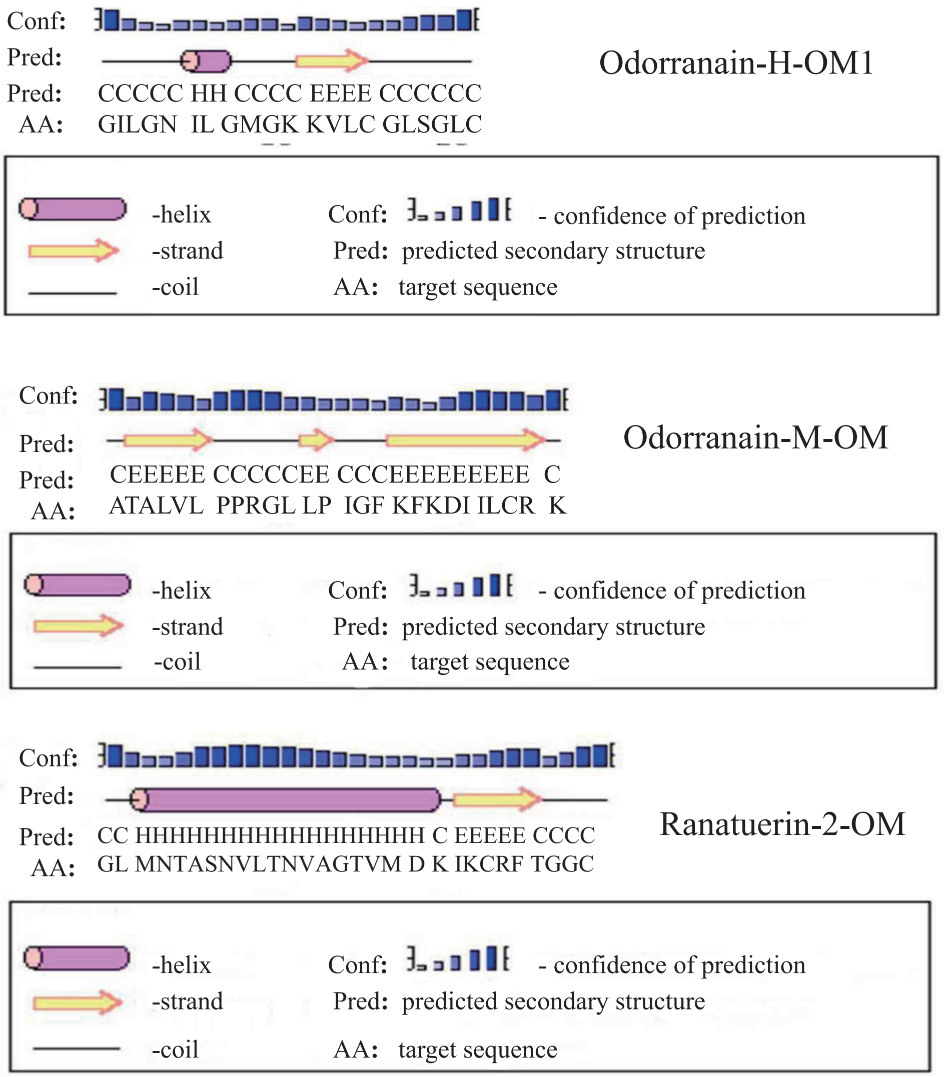
Conf:
Pred:
Pred: CCCCC HH CCCC EEEE CCCCCC
AA: GILGN IL GMGK KVLC GLSGLC
Odorranain-H-OM1
-helix
-strand
-coil
Conf: - confidence of prediction
Pred: predicted secondary structure
AA: target sequence
Conf:
Pred:
Pred: CEEEEE CCCCCEE CCCEEEEEEEEE C
AA: ATALVL PPRGL LP IGF KFKDI ILCR K
Odorranain-M-OM
-helix
-strand
-coil
Conf: - confidence of prediction
Pred: predicted secondary structure
AA: target sequence
Conf:
Pred:
Pred: CC HHHHHHHHHHHHHHHH C EEEEE CCCC
AA: GL MNTASNVLTNVAGTVM D K IKCRF TGGC
Ranatuerin-2-OM
-helix
-strand
-coil
Conf: - confidence of prediction
Pred: predicted secondary structure
AA: target sequence

Figure 3 Secondary structure prediction of odorranain-H-OM1, odorranain-M-OM and ranatuerin-2-OM.

odorranain-H-OM1 didn't exhibit stable secondary structure, which was mainly composed of random coil. In contrast, odorranain-M-OM exhibited a dominate strand structure and ranatuerin-2-OM was the mixture of helix and strand.

3.2 Antimicrobial activity As listed in Table 2, odorranain-H-OM1 exhibited relatively broad antimicrobial activity against the tested microorganisms. *Proteus mirabilis* clinical strain (Gram-negative bacteria), *Escherichia coli* clinical strain (Gram-negative bacteria), *Enterococcus faecium* clinical strain (Gram-positive bacteria) and *Candida glabrata* clinical strain (Fungi) were sensitive to odorranain-H-OM1, with MIC values of 18.75, 9.38, 9.38 and 9.38 µg/ml, respectively. Among which, *Escherichia coli* clinical strain, *Enterococcus faecium* clinical strain and *Candida glabrata* clinical strain were ampicillin-resistant. However, odorranain-M-OM and ranatuerin-2-OM showed no activity against all of the ten microorganisms.

3.3 Anti-oxidant activity At the concentration of 80 µg/ml, odorranain-H-OM1, odorranain-M-OM and ranatuerin-2-OM showed slight DPPH radical scavenging activity, with I % values of 5.71 %, 10.36 % and 7.04 %, respectively. However, at the same concentration, the three peptides exhibited strong ABTS^{+} radical scavenging activities, with I% values of 21.59 %, 30.82 % and 14.97 %, respectively. Moreover, as shown in Figure 4, odorranain-M-OM showed a concentration-dependent ABTS^{+} radical scavenging activity. At the concentration of 320 µg/ml, it showed the highest ABTS^{+} radical scavenging rate of 51.90 %.

3.4 Erythrocyte hemagglutinating activity Lectins are

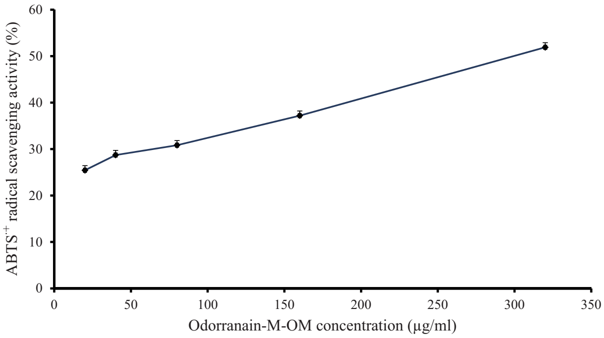

Figure 4 ABTS$^{•+}$ radical scavenging activity of odorranain-M-OM. Serial concentrations of odorranain-M-OM were incubated with ABTS$^{•+}$ solution at room temperature for 10 min and absorbance of the mixtures at 734 nm was measured. Results represent mean values of triplicate experiments.

glycan-binding proteins that can specifically recognize glycan structures and have been identified from a wide variety of organisms (Fujii *et al.*, 2012). Several kinds of lectins have been reported to be important pathogen associated molecular patterns (PAMPs) participating in the host immune responses against external/environmental stimuli (Kim *et al.*, 2006; Venier *et al.*, 2011). In the present study, ranatuerin-2-OM (200 μg/ml) showed strong hemagglutinating activity to human intact erythrocytes, and the activity didn't depend on the presence of Ca^{2+} (Figure 5). In contrast, odorranain-H-OM1 and odorranain-M-OM didn't exhibit any hemagglutinating activity.

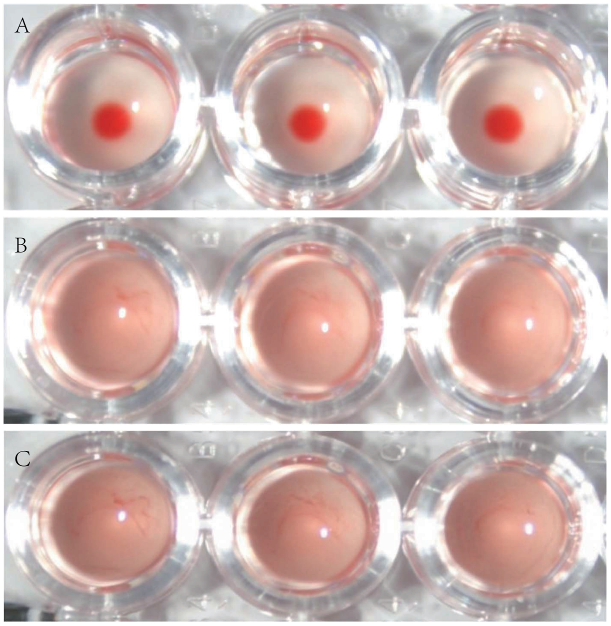

Figure 5 Hemagglutinating activity of ranatuerin-2-OM. Ranatuerin-2-OM solution was mixed with human erythrocyte solution in U-well microtiter plate and remained at room temperature for 45 min before observation. (A) negative control; (B) ranatuerin-2-OM dissolved in TBS buffer; (C) ranatuerin-2-OM dissolved in TBS+Ca^{2+} buffer.

Table 2 Antimicrobial activities of the three selected HDPs.

Microorganisms	MIC (μg/ml)			
	Odorranain-H-OM1	Odorranain-M-OM	Ranatuerin-2-OM	Ampicillin
Gram-negative bacteria				
Proteus mirabilis clinical strain	18.75	ND	ND	75
Pseudomonas aeruginosa ATCC27853	ND	ND	ND	37.5
Escherichia coli ATCC25922	ND	ND	ND	75
Escherichia coli clinical strain	9.38	ND	ND	ND
Gram-positive bacteria				
Staphylococcus aureus ATCC2592	ND	ND	ND	4.69
Staphylococcus epidermidis clinical strain	ND	ND	ND	ND
Enterococcus faecium clinical strain	9.38	ND	ND	ND
Fungi				
Candida albicans ATCC2002	ND	ND	ND	4.69
Candida albicans clinical strain	ND	ND	ND	ND
Candida glabrata clinical strain	9.38	ND	ND	ND

MIC: minimal inhibitory concentration. These concentrations represent mean values of three independent experiments performed in duplicates.

4. Discussion

Previous research identified 72 AMPs belonging to 21 families from the skin of *O. margaretae*, 62 of these were novel (Figure 6) (Yang *et al.*, 2012). Besides, 19 AMPs belonging to 13 families were also identified by H. Wang and J. Liu and were uploaded to the UniProt (http://www.uniprot.org/uniprot/?query=peptides+Odorrana+margaretae) (Figure 7). Among them, 8 had already been discovered by Yang *et al.* (2012), although they were designated in different names (esculentin-2-MG=esculentin-2-OMar1, brevinin-2E-MG=brevinin-2-OMar1, odorranain-A-MG1=odorranain-A-OMar3, odorranain-A-MG2=odorranain-A-OMar1, odorranain-P1b=brevinin-1-OMar6, ranatuerin-2-MG=andersonin-W-OMar2, nigrosin-MG2=nigrosin-OMar2, nigrosin-MG3=nigrosin-OMar3). Unfortunately, the sampling location of H. Wang and J. Liu remains unknown. In the present study, 17 HDPs belonging to 14 families were further discovered from the skin of *O. margaretae*. But only one (brevinin-1-Omar5) of the 17 HDPs discovered in the present study has been identified previously (Yang *et al.*, 2012). It is the first time for the other 16 HDPs to be discovered from *O. margaretae*. However, most of the novel HDPs in the present study exhibited high sequence similarity with the HDPs identified previously. We made an alignment of these HDPs to the similar peptides identified by (Yang *et al.*, 2012) and H. Wang, J. Liu. As shown in Figure 8, ishikawain-7-OM exhibited high similarity with rotodorin-F-OMar and margaretaein-A1, and actually they belonged to the same family. Similarly, odorranain-C and brevinin-2, odorranain-H and nigrocin were also the same HDPs family. Except for the 8 novel HDPs in Figure 8 (ishikawain-7-OM, odorranain-A-OM, odorranain-C-OM1-2, odorranain-H-OM1-2, odorranain-M-OM and odorranain-Q-OM), the other 3 novel HDPs (ranatuerin-2-OM, palustrin-OM and andersonin-Q-OM) identified in the present study didn't exhibit any sequence

Brevinin-1-OMar
1 FFPLFASLAANFLPKLFCKIARKC
2 FFPLLAGLAANFLPKLFCKIARKC
3 FFSLLAGLAANFLPKLFCKIARKC
4 FFPLLAGLAANFLPKLFCKIARKY
5 VIPFVASVAAEMMQHVYCAASKRC
6 VIPFVASVAAEMMQHVYCAASKKC
7 VIPFVASVAAEMMQHVYCAASIKC
Brevinin-2-OMar
1 GLLDTFKNLALNAAKSAGVSVLNSLSCKLSKTC
2 GLLDTFKNLALNAAKSAGVSVLNSLSCKLPKTC
3 GVLGTVKNLLIGAGKSAAQSVLKGLACKLSNDC
Nigrocin-OMar
1 GLISGILGAGKKVLCGLSGLC
2 GLLSGILGAGKNIVCGLSGLC
3 GLLSGVLGVGKKIVCGLSGLC
4 GLISGILGARKKVLCGLSGLC
5 GLLSGILSAGKNIVCGLSGLC
6 GIFGKILGVGKKVLCGLSGMC
7 GLLSGVLGVGKKVLCGLSGLC
8 GLSSGILGAGKNIVCGLSGLC
9 GLLSGILGAGKKVLCGLSGLC
10 GILSGLLGAGKKIVCGLSGLC
11 GLLSGILGAGKNIVCGLSGLY
12 GLLSGILGAGKNIVCGLSWLC
13 GPISGILGAGKKVLCGLSGLC
14 GLISGILGAGKNIVCGLSGLC
15 GLLGGILGAGKNIVCGLSGLC
16 GLLSGVLGVGKKVLCGLSGMC
17 GIFGKILGVGKKVLCGLSGLC
18 GLLSGVLGVGKKVLCGLSGQC
19 GLLIGVLGVGKKVLCGLSGLC
20 GLFGGVLGVGKKIVCGLSGLC
21 GIFGKILGAGKKVLCGLSGLC
22 GLLSGVLGVGKKVVCGLSGLC
23 GIFGKILGVGKNIVCGLSGLC
24 GLLSGVLGVGKSIVCGLSGLC
25 GIFGKILGVGKKVLWGLSGLC
Margaratain-A
1 VTPPWARIYYGCAKA
2 VIPPWARIYYG
Margaratain-B
1 FFSTSCRSGC
Margaratain-C
1 GMLKWKNDFFHFLQVWLLISCQNYFVK
2 GMLKWKNDFFHFLQVWLLISCQNYFVK
Margaratain-D
1 GLFSKFAGEGIKNLLFKGVQAHRQGSWHGCDQNWDRRCRL
Esculentin-1-OMar
1 GLFSKFAGKGIKNFLIKGVKHIGKEVGMDVIRTGIDVAGCKIKGEC
2 GLFSKFAGRGIKNFLIKGVKHIGKEVGMDVIRTGIDVAGCKIKGEC
3 GLFSKFAEKGIKNLLFKGVKHIGKEVGMDVIRTGIDVAGCKIKGEC
4 GLFSKFAGKGIKNLLFKGVKHIGKEVGMDVIRTGIDVAGCKIKGEC
5 GLFSKFAGKGIKNFLIKGVKHIGKEVGMDVIRTGIDVAGCKIKGVC
6 GLFSKFAGKGIKNFLIKGVKHIGKEVGMDVIRTGVDVAGCKIKGEC
7 GLFSKFAGKGIKNFLIKGVKHIGKEVGMDVIRTGIDVAGCKVKGEC
8 GLFSKFAGKGIKNFLIKGVKHIGKEVGMDVIRTGIDVAGGKIKGEC
9 GLFSKFAGKGIKNFLIKGVKHIGKEVGMDVVRTGIDVAGCKIKGEC
10 GLFSKFAGRGIKNFLIKGVKHTGKEVGMDVIRTGIDVAGCKIKGEC
Esculentin-2-OMar
1 GLFTLIKGAAKLIGKTVAKEAGKTGLEFMACKITNQC
2 GLFTLIKGAAKLIGKTVAKEAGKTGLEFMACRITNQC
3 GVFRLIKGAPKCIGKDGAKEARKSGQEFMGCKITKQC
4 GLFTLIKGAAKLIGKTVAKEAG
Odorranain-A-OMar
1 -VVKCSYREGSPDSRCK
2 -VVKCSCREGSPDSRCK
3 RVFKCYEPDSRGFQVCE
Odorranain-B-OMar
1 AALKGCWTKSIPPKPCFGKR
2 AALRGCWTKSIPPKPCSGKR
3 AALKGCWTKSIPSKPCFGKR
Odorranain-K-OMar
1 GFFPLIKGPAKLIAKTVAKKPAKTGLEFML
Odorranain-M-Omar
1 ATALGLSSRGLLPIGFMFKDTIRCRKD
Odorranain-O-OMar
1 AVPLIYNRPGIYVTKRPKGK
Odorranain-Q-OMar
1 APFRMWYMYHKLKDMEPKPMA
Odorranain-S-OMar
1 FLPPSPWKETFRTS
Odorranain-U-OMar
1 GCSRWIIGINGQICRD
Odorranain-W-OMar
1 GLISGILGRGRKYYVDLAGCAKA
Rotodorin-F-OMar
1 CGYKHGRANCGRG
Andersonin-W-OMar
1 AVNIPFKIHFRCKAAFC
2 AVNIPFKVHFRCKAAFC
Rotodorin-C-OMar
1 IVPNCNYKFSGANCLGKERYMKWKSPDVVYHLAK

Figure 6 The 72 AMPs identified by Yang *et al*. (2012) in the skin of *O. margaretae* collected in the Hunan province.

Esculentin-1-MG
1 GLFSKFAGKGIKNFLFKGVKHIGKEVGMDVIRTGIDVAGCKIKGEC
2 GLFSKFAGKGIKNFLIKGVKHIGKEVGMDVIRTGIDVAGCKIKGEC
Esculentin-2-MG
1 GLFTLIKGAAKLIGKTVAKEAGKTGLEFMACKITNQC
Brevinin-1E-MG
1 FLPLLAGLAANFLPKLFCKITRKC
2 FLPFLAGLAANFLPKLFCKITRKC
Brevinin-2E-MG
1 GLLDTFKNLALNAAKSAGVSVLNSLSCKLSKTC
Odorranain-A-MG
1 RVFKCYEPDSRGFQVCE
2 VVKCSYREGSPDSRCK
Odorranain-C-MG
1 GVLGTVKNLLIGAGKSAAQSVLKGLACKLSNDC
2 GVLGTVKNLLIGAGKSAAQSVLKGLACKLSNNC
Odorranain-F-MG
1 GFMDTAKNVAKNVAVTLLDNLRCKITKAC
Odorranain-M-MG
1 ATAWDFGPHGLRPIRPIRIRPLCGKDKS
Odorranain-P1b
1 VIPFVASVAAEMMQHVYCAASKKC
Odorranain-U-MG
1 GCSRWIIGINGQVCRD
Margaretaein-A
1 CGYRHGRANCGRG
Ranatuerin-2-MG
1 AVNIPFKVHFRCKAAFC
Nigrosin-MG
1 GLISGILGAGKKVCGLSGLC
2 GLLSGILGAGKNIVCGLSGLC
3 GLLSGVLGVGKKIVCGLSGLC

Figure 7 The 19 HDPs identified by H. WANG and J. LIU in the skin of *O. margaretae*.

similarity with the HDPs identified by Yang *et al.* and H. Wang and J. Liu.

Geographically separated populations of frogs usually encounter diverse environmental factors, such as microbial flora, temperature, chemicals and stress. It is no doubt that the difference in environmental microbial flora can result in diverse threats to frogs. As a result, it is rational to observe intraspecies variation in the constituents and activities of HDPs in geographically separated populations of frogs. Previous study about the expressed AMPs repertoire of northern leopard frog (*Rana pipiens*) partly confirmed the hypothesis (Tennessen *et al.*, 2009). Environmental chemicals, such as pesticides, have been reported can alter immune defenses of amphibians and their susceptibility to pathogens (Rollins-Smith *et al.*, 2011). Exposure to sublethal concentrations of carbaryl in foothill yellow-legged frogs (*Rana boylii*) significantly reduced the skin-peptide defense system (Davidson *et al.*, 2007). Furthermore, immune responses of frogs also can be impaired by low temperature. Prevalence of infections of frogs increases in cooler seasons (Rollins-Smith *et al.*, 2011; Woodhams *et al.*, 2008). Stresses in the life of frogs, such as metamorphosis, intensive breeding and food deprivation, have been reported can result in natural elevation of corticosteroid hormones, immune suppression, and greater susceptibility to bacterial infections (Crespi and Denver, 2005; Jolivet Jaudet and Leloup Hatey, 1984; Mendonca *et al.*, 1985). Pharmacological concentrations of corticosterone can completely inhibit renewal of amphibian skin peptides following norepinephrine-induced depletion of the peptides in adult *Xenopus laevis* (Rollins-Smith *et al.*, 2011). In the previous study, Yang *et al.* (2012) collected the frogs in the Hunan province, and generally the sampling period should be April–August, in which *Odorrana* frogs breed and are hyperactive. However, in the present study, we collected the frogs in the southern Guizhou province in October. The environmental differences between the sampling locations and the different sampling times should be responsible for the distinct HDPs expression profile of *O. margaretae*. Except for the environmental differences, genetic divergence among *O. margaretae* populations may be another possible reason for the distinct HDPs expression profile. It has been reported that high diversity among alleles at a single AMP locus (brevinin-1) exists within a single frog species, both within populations and between populations (Tennessen and Blouin, 2008). *O. margaretae* is a widely distributed species in the southern provinces of China. After a long period of independent evolution process, *O. margaretae* frogs of different populations might evolve high genetic divergence. As a result, it is rational to observe great HDPs expression diversity between *O. margaretae* populations collected from different locations. In the previous study, three AMPs (margaratain-A1, B1 and C1) identified by Yang *et al.* (2012) from the skin of *O. margaretae* were synthesized and their *in vitro* activities were studied (Yang *et al.*, 2012). The three AMPs did not exhibit antimicrobial activity, but showed obvious anti-oxidant activity. Besides, margaratain-B1 showed potential inhibitory effect on TNF-α production induced by LPS. Furthermore, margaratain-A1 and B1 could obviously increase the release of IL-8. The results implied that margaratain-A1, B1 and C1 may play immunomodulating effects in host immune responses of *O. margaretae* and should be designated as HDPs, rather than AMPs. In the present study, three novel HDPs (odorranain-H-OM1, odorranain-M-OM and ranatuerin-2-OM) were also chemically synthesized and functionally analyzed. The three HDPs exhibited diverse activities. Odorranain-H-OM1 showed direct antimicrobial activity against Gram-positive and Gram-negative bacteria and fungi. Odorranain-M-OM exhibited concentration-dependent anti-oxidant activity. Ranatuerin-2-OM showed lectin-like activity and can strongly hemagglutinate

Ishikawain-7-OM	CGYRHGRAHCGRG	(the present study)
Rotodorin-F-Omar	CGYKHGRANCGRG	(Yang *et al*., 2012)
Margaretaein-A1	CGYRHGRANCGRG	(H. Wang and J. Liu)

Odorranain-A-OM	RVFKCYKPDSRGFQICE	(the present study)
Odorranain-A-Omar1	-VVKCSYREGSPDSRCK	(Yang *et al*., 2012; H. Wang and J. Liu)
Odorranain-A-OMar2	-VVKCSCREGSPDSRCK	(Yang *et al*., 2012)
Odorranain-A-Omar3	RVFKCYEPDSRGFQVCE	(Yang *et al*., 2012; H. Wang and J. Liu)

Odorranain-C-OM1	SVMGTVKDLLIGAGKSAAQSVLKALSCKLSKDC	(the present study)
Odorranain-C-OM2	SVMGTVKDLLIGAGKSAAQSVLKSLSCKLSNDC	(the present study)
Brevinin-2-OMar1	GLLDTFKNLALNAAKSAGVSVLNSLSCKLSKTC	(Yang *et al*., 2012; H. Wang and J. Liu)
Brevinin-2-OMar2	GLLDTFKNLALNAAKSAGVSVLNSLSCKLPKTC	(Yang *et al*., 2012)
Brevinin-2-OMar3	GVLGTVKNLLIGAGKSAAQSVLKGLACKLSNDC	(Yang *et al*., 2012)
Odorranain-C-MG1	GVLGTVKNLLIGAGKSAAQSVLKGLACKLSNDC	(H. Wang and J. Liu)
Odorranain-C-MG2	GVLGTVKNLLIGAGKSAAQSVLKGLACKLSNNC	(H. Wang and J. Liu)

Odorranain-H-OM1	GILGNILGMGKKVLCGLSGLC	(the present study)
Odorranain-H-OM2	GILGNIVGMGKKIVCGLSGLC	(the present study)
Nigrocin-OMar1	GLISGILGAGKKVLCGLSGLC	(Yang *et al*., 2012)
Nigrocin-OMar2	GLLSGILGAGKNIVCGLSGLC	(Yang *et al*., 2012; H. Wang and J. Liu)
Nigrocin-OMar3	GLLSGVLGVGKKIVCGLSGLC	(Yang *et al*., 2012; H. Wang and J. Liu)
Nigrocin-OMar4	GLISGILGARKKVLCGLSGLC	(Yang *et al*., 2012)
Nigrocin-OMar5	GLLSGILSAGKNIVCGLSGLC	(Yang *et al*., 2012)
Nigrocin-MG1	GLISGILGAGK-KVCGLSGLC	(H. Wang and J. Liu)

Odorranain-M-OM	ATALVLPPRGLLPIGFKFKDIILCRKD	(the present study)
Odorranain-M-Omar	ATALGLSSRGLLPIGFMFKDTIRCRKD	(Yang *et al*., 2012)

Odorranain-Q-OM	APFRMWYMYHKLKDMEPKPVA	(the present study)
Odorranain-Q-Omar	APFRMWYMYHKLKDMEPKPMA	(Yang *et al*., 2012)

Figure 8 A comparison of the primary structures of the HDPs identified in the present study with the HDPs identified by Yang *et al*. (2012) and H. WANG and J. LIU in the skin of *O. margaretae*.

human intact erythrocytes. The results of functional analysis in the previous and our present study imply that HDPs from *O. margaretae* skin may play different roles in host defense reactions and provide templates for the development of novel anti-infective agents.

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References

Auvynet C., Rosenstein Y. 2009. Multifunctional host defense peptides: antimicrobial peptides, the small yet big players in innate and adaptive immunity. FEBS J, 276: 6497–6508

Bevins C. L., Zasloff M. 1990. Peptides from frog skin. Annu Rev Biochem, 59: 395–414

Choi K. Y., Chow L. N., Mookherjee N. 2012. Cationic host defence peptides: multifaceted role in immune modulation and inflammation. J Innate Immun, 4: 361–370

Conlon J. M. 2011. Structural diversity and species distribution of host-defense peptides in frog skin secretions. Cell Mol Life Sci, 68: 2303–2315

Conlon J. M., Kolodziejek J., Nowotny N. 2004. Antimicrobial peptides from ranid frogs: taxonomic and phylogenetic markers and a potential source of new therapeutic agents. Biochim Biophys Acta, 1696: 1–14

Crespi E. J., Denver R. J. 2005. Roles of stress hormones in food intake regulation in anuran amphibians throughout the life cycle. Comp Biochem Physiol A Mol Integr Physiol, 141: 381–390

Davidson C., Benard M. F., Shaffer H. B., Parker J. M., O'Leary C, Conlon J. M., Rollins-Smith L. A. 2007. Effects of chytrid and carbaryl exposure on survival, growth and skin peptide defenses in foothill yellow-legged frogs. Environ Sci Technol, 41: 1771–1776

Fujii Y., Dohmae N., Takio K., Kawsar S. M., Matsumoto R., Hasan I., Koide Y., Kanaly R. A., Yasumitsu H., Ogawa Y., Sugawara S., Hosono M., Nitta K., Hamako J., Matsui T., Ozeki Y. 2012. A lectin from the mussel *Mytilus galloprovincialis* has a highly novel primary structure and induces glycan-mediated cytotoxicity of globotriaosylceramide-expressing lymphoma cells. J Biol Chem, 287: 44772–44783

Hancock R. E., Sahl H. G. 2006. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat Biotechnol, 24: 1551–1557

He W. Y., Feng F. F., Huang Y., Guo H. H., Zhang S. Y., Li Z., Liu J. Z., Wang Y. P., Yu H. N. 2012. Host defense peptides in skin secretions of *Odorrana tiannanensis*: Proof for other survival strategy of the frog than merely anti-microbial. Biochimie, 94: 649–655

Jolivet Jaudet G., Leloup Hatey J. 1984. Variations in aldosterone and corticosterone plasma levels during metamorphosis in *Xenopus laevis* tadpoles. Gen Comp Endocrinol, 56: 59–65

Kim Y. M., Park K. I., Choi K. S., Alvarez R. A., Cummings R. D., Cho M. 2006. Lectin from the Manila clam *Ruditapes philippinarum* is induced upon infection with the protozoan parasite *Perkinsus olseni*. J Biol Chem, 281: 26854–26864

Li J. X., Wu J., Wang Y. P., Xu X. Q., Liu T. G., Lai R., Zhu H. 2008a. A small trypsin inhibitor from the frog of *Odorrana grahami*. Biochimie, 90: 1356–1361

Li J. X., Wu H. B., Hong J., Xu X. Q., Yang H. L., Wu B. X., Wang Y. P., Zhu J. H., Lai R., Jiang X. G., Lin D. H., Prescott M. C., Rees H. H. 2008b. Odorranalectin is a small peptide lectin with potential for drug delivery and targeting. PLoS ONE, 3: e2381

Li J. X., Xu X. Q., Xu C. H., Zhou W. P., Zhang K. Y., Yu H. N., Zhang Y. P., Zheng Y. T., Rees H. H., Lai R., Yang D. M., Wu J. 2007. Anti-infection peptidomics of amphibian skin. Mol Cell Proteomics, 6: 882–894

Liu C. B., Hong J., Yang H. L., Wu J., Ma D. Y., Li D. S., Lin D. H., Lai R. 2010. Frog skins keep redox homeostasis by antioxidant peptides with rapid radical scavenging ability. Free Radic Biol Med, 48: 1173–1181

Lu Z. K., Zhai L., Wang H., Che Q. L., Wang D., Feng F. F., Zhao Z. M., Yu H. N. 2010. Novel families of antimicrobial peptides with multiple functions from skin of Xizang plateau frog, *Nanorana parkeri*. Biochimie, 92: 475–481

Mechkarska M., Ahmed E., Coquet L., Leprince J., Jouenne T., Vaudry H., King J. D., Conlon J. M. 2010. Antimicrobial peptides with therapeutic potential from skin secretions of the Marsabit clawed frog X*enopus borealis* (Pipidae). Comp Biochem Physiol C Toxicol Pharmacol, 152: 467–472

Mendonca M. T., Licht P., Ryan M. J., Barnes R. 1985. Changes in hormone levels in relation to breeding behavior in male bullfrogs (*Rana catesbeiana*) at the individual and population levels. Gen Comp Endocrinol, 58: 270–279

Mookherjee N., Hancock R. E. 2007. Cationic host defence peptides: innate immune regulatory peptides as a novel approach for treating infections. Cell Mol Life Sci, 64: 922–933

Rollins-Smith L. A., Ramsey J. P., Pask J. D., Reinert L. K., Woodhams D. C. 2011. Amphibian immune defenses against chytridiomycosis: impacts of changing environments. Integr Comp Biol, 51: 552–562

Tennessen J. A., Blouin M. S. 2008. Balancing selection at a frog antimicrobial peptide locus: fluctuating immune effector alleles? Mol Biol Evol, 25: 2669-2680

Tennessen J. A., Woodhams D. C., Chaurand P., Reinert L. K., Billheimer D., Shyr Y., Caprioli R. M., Blouin M. S., Rollins-Smith L. A. 2009. Variations in the expressed antimicrobial peptide repertoire of northern leopard frog (*Rana pipiens*) populations suggest intraspecies differences in resistance to pathogens. Dev Comp Immunol, 33: 1247–1257

Venier P., Varotto L., Rosani U., Millino C., Celegato B., Bernante F., Lanfranchi G., Novoa B., Roch P., Figueras A., Pallavicini A. 2011. Insights into the innate immunity of the Mediterranean mussel *Mytilus galloprovincialis*. BMC Genomics, 12: 69

Woodhams D. C., Alford R. A., Briggs C. J., Johnson M., Rollins-Smith L. A. 2008. Life-history trade-offs influence disease in changing climates: strategies of an amphibian pathogen. Ecology, 89: 1627–1639

Yang H. L., Wang X., Liu X. H., Wu J., Liu C. B., Gong W. M., Zhao Z. Q., Hong J., Lin D. H., Wang Y. Z., Lai R. 2009. Antioxidant peptidomics reveals novel skin antioxidant system. Mol Cell Proteomics, 8: 571–583

Yang X. W., Lee W. H., Zhang Y. 2012. Extremely abundant antimicrobial peptides existed in the skins of nine kinds of Chinese odorous frogs. J Proteome Res, 11: 306–319

Yeung A. T., Gellatly S. L., Hancock R. E. 2011. Multifunctional cationic host defence peptides and their clinical applications. Cell Mol Life Sci, 68: 2161–2176